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Antioxidant Activity of Zein Hydrolysates in a Liposome System and the Possible Mode of Action†

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Maize zein was hydrolyzed for 0.5−5 h by alcalase or papain. Protein solubility increased ($P < 0.05$) with the degree of hydrolysis (DH) and was higher for alcalase-hydrolyzed zein than for papain-hydrolyzed zein. The zein hydrolysates with both enzymes consisted mostly of small peptides or amino acids nondetectable by 15% acrylamide gel electrophoresis. Alcalase-hydrolyzed zein exhibited a stronger ($P < 0.05$) antioxidant activity than papain-hydrolyzed zein, as indicated by peroxide and thiobarbituric acid-reactive substance values in a liposome-oxidizing system. Zein hydrolysates possessed strong Cu^{2+} chelation ability and marked reducing power, both of which were accentuated with hydrolysis time. The protein hydrolysates also showed strong radical-scavenging ability, which was not influenced by hydrolysis time. The antioxidant activity of alcalase-hydrolyzed zein at some specific low concentrations was close or comparable to those of butylated hydroxyanisole, α-tocopherol, and ascorbate. Although intact zein displayed an antioxidative effect, it was far less potent than hydrolyzed zein. The results demonstrated that enzyme-hydrolyzed zein can act as a metal ion chelator or a hydrogen donor, as well as a radical stabilizer to inhibit lipid oxidation. The effectiveness of the protein hydrolysates appeared to depend on both the concentration and the peptide/amino acid composition of the soluble protein fraction.

KEYWORDS: Zein; protein hydrolysates; antioxidants; lipid oxidation

INTRODUCTION

Corn (maize) is a major crop in the world. In the food industry, mature corn kernels are used to produce oil and starch. However, corn protein, which is obtained as a byproduct of corn starch and oil production, is rarely utilized in human foods as a food ingredient (*1*). Reasons for the hindrance are largely related to the poor functional properties of the major protein component, that is, zein (*2*). Zein, a prolamine protein, has a high concentration of nonpolar amino acids such as leucine, proline, and alanine (*1*), and it is also characterized by very low concentrations of lysine and tryptophan, which account for its low nutritional quality and low solubility in aqueous solutions (*3*). On the other hand, zein has been widely used for films and coatings because of its hydrophobic properties (*4*, *5*).

Autoxidation of fats and oils in foods is responsible for flavor deterioration as well as nutrient loss. Synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), may be added to food products to retard lipid oxidation. However, concerns about the long-term safety and negative consumer perception of synthetic antioxidants have led to an increasing demand for natural antioxidants in food. Among the widely used natural antioxidants in foods today are tocopherols, rosmarinic acid, catechins, and various mixed phenolic plant extracts (*6*). Furthermore, a large number of plant- and animal-derived proteins and protein hydrolysates have been found to possess significant antioxidant activity against the peroxidation of lipids and fatty acid. These include, for example, hydrolyzed whey protein (*7*−*9*), soy protein (*10*−*12*), fish protein (*13*, *14*), egg proteins (*15*, *16*), pork protein (*17*, *18*), elastin (*19*), and gelatin (*20*, *21*).

Despite these previous investigations, little is known about the antioxidant effect of zein hydrolysates prepared by enzymatic treatments. The objective of this study was to determine the antioxidant activity of zein hydrolysates prepared by alcalase and papain. Moreover, the ferric-reducing power, radical cation-scavenging activity, and metal ion-chelating capacity of selected hydrolysates and their efficacy in comparison with several common antioxidants were determined to elucidate their possible antioxidant mechanism.

MATERIALS AND METHODS

Materials. Zein was purchased from Freeman Industries LLC (Tuckahoe, NY). The dry zein powder contained 92% protein on a dry weight basis. Alcalase (endoproteinase from *Bacillus licheniformis,* 2.4 AU/g) was obtained from Novozymes North America Inc. (Franklinton, NC). Papain (P-3375, 1.8 units/mg of solid), ferrous sulfate ($FeSO_4$·

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$7H_2O$), 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), Trolox, BHA, α-tocopherol, sodium ascorbate, and soybean phosphatidylcholine were purchased from Sigma Chemical Co. (St. Louis, MO). Thiobarbituric acid (TBA) was obtained from ICN Biomedicals Inc. (Aurora, OH), and sodium dodecyl sulfate (SDS) was purchased from Bio-Rad Laboratories (Hercules, CA). All other chemicals were purchased from Fisher Scientific (Fair Lawn, NJ) and were of at least reagent grade.

Preparation of Zein Hydrolysates. Zein (2, 3, and 5% w/v aqueous solution) was hydrolyzed with alcalase at 50 °C for 0.5, 1, 2, 3, 4, and 5 h and with papain at 37 °C for 0.5, 1, 2, 3, 4, 5, 8, and 24 h. The enzyme to zein substrate ratio was 2:100. The pH of the zein solutions was adjusted to the optimal values for the specific proteases (pH 9.0 for alcalase; pH 8.0 for papain) before hydrolysis was initiated, and it was readjusted to the optimal value every 30 min during hydrolysis with 1 M NaOH. After hydrolysis, the pH of the broths was brought to 7.0, and the solutions were then heated at 95 °C for 5 min to inactivate the enzymes. The hydrolysates were freeze-dried (Dura-Dry MP freeze-dryer, FTS Systems, Inc., Ridge, NY), pulverized, placed in sealed bags, and stored at 4 °C before use. The zein hydrolysate powders (as well as the nonhydrolyzed zein) contained yellow pigments. To eliminate possible color interferences with the following chemical analyses, aqueous suspensions of intact zein and zein hydrolysates were decolored by mixing with an equal volume of chloroform. The organic phase, which contained the extracted pigments, was removed.

Degree of Hydrolysis (DH). The DH of hydrolyzed protein was determined using a pH-stat method (*22*). The DH was calculated on the basis of the equations

$$\mathrm{DH} = (h/h_{\mathrm{tot}}) \times 100\%$$

$$h = B \times N_{\mathrm{b}} \times 1/\alpha \times 1/\mathrm{MP}$$

where B = base consumption (mL), N_{b} = concentration of base (1 M NaOH), $1/\alpha$ = calibration factors for pH-stat ($1/\alpha = 1.01$ for alcalase; $1/\alpha = 1.20$ for papain), MP = mass of protein (g), h = hydrolysis equivalents, that is, the amount of peptide bonds cleaved during hydrolysis, which is expressed as millimole equivalents per gram of protein (mmol/g of protein), and h_{tot} = total amount of peptide bonds in the protein substrate, which can be determined from the amino acid composition. For zein, $h_{\mathrm{tot}} = 9.2$ mmol/g of protein (*22*).

Protein Solubility. Freeze-dried hydrolysates of zein were suspended (3%) in distilled water and centrifuged at 1800*g* for 5 min. The protein concentration of the supernatants was measured according to the biuret method (*23*). Protein solubility (percent) was defined as the protein concentration in the supernatant divided by the protein concentration of the original suspension and then multiplied by 100.

Electrophoresis. Nonhydrolyzed zein and its hydrolysates were subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (*24*) with slight modifications. The SDS-PAGE was run with a 15% acrylamide resolving gel and a 3% acrylamide stacking gel using a Mini-Protean 3 cell electrophoresis system (Bio-Rad Laboratories). Protein samples (1 mL at 4 mg of protein/mL) were dissolved in 1 mL of sample buffer (4% SDS, 20% glycerol, 10% β-mercaptoethanol, and 0.125 M Tris-HCl, pH 6.8), heated at 100 °C for 3 min, and centrifuged at 1800*g* to remove particulates. Aliquots of 20 μL of the supernatants were loaded in each well on the gel. A molecular weight (MW) standard, composed of a cocktail of proteins (6.5−200 kDa) (Bio-Rad Laboratories), was also run. The MWs of individual protein bands in samples were estimated from the regression line of the protein migration distance versus the logarithm of the MW. To quantify the protein bands, the polyacrylamide gels were scanned with an LKB Ultrascan XL laser densitometer (Pharmacia LKB Biotechnology AB, Uppsala, Sweden), and the areas of corresponding peaks were integrated with the software built in the densitometer.

Amino Acids Analysis. Aliquots of 1.0 mL of whole zein (predissolved in 65% ethanol) and the supernatants of zein hydrolysates from 0.5, 1, 2, and 4 h of alcalase hydrolysis were mixed with 0.25 mL of 2% phenol solution and 0.5 mL of performic acid. After incubation at 20 °C for 4 h, 0.2 mL of 16.8% sodium pyrosulfite was added. The samples were subsequently digested in a sealed glass tube with 9 mL of 6.7 N HCl at 110 °C for 24 h. The whole digests were transferred into 50-mL volumetric flasks, mixed with 9 mL of 6 M NaOH, and then brought to volume (50 mL) with 0.02 N HCl. The sample solutions were subjected to amino acid analysis using a model L-8800 amino acid analyzer (Hitachi Co., Tokyo, Japan) equipped with a Hitachi 2622 cation exchange column (4.6 mm × 60 mm) for amino acid separation. Postcolumn reaction with ninhydrin yielded amino acid derivatives, the absorbances of which were measured at 570 and 440 nm. The concentrations of the specific amino acids were determined from their respective absorption intensities, which were calibrated to the known concentrations of amino acid standards.

Lipid Oxidation. Liposomes were prepared from soybean phosphatidylcholine according to the method of Decker and Hultin (*25*) with some modifications. The soybean phosphatidylcholine was dispersed (0.2 mg/mL) in 0.12 M KCl and 5 mM histidine buffer (pH 6.8) by homogenization followed by sonication at 4 °C for 45 min with a model 300 Branson SonifierSonic Dismembrator (Fisher Scientific). To measure the antioxidant activity of zein hydrolysates, a series of mixed solutions of 5 mL of liposome and 1 mL of zein hydrolysates (0.5−24 h hydrolysates at 0.5, 1, 2, or 3% protein concentrations) were prepared. Nonhydrolyzed zein was predissolved in 65% ethanol before mixing with the liposome solution. The control solution was prepared by mixing 1 mL of water instead of 1 mL of protein solution with 5 mL of liposome. For comparison, 0.01% BHA and α-tocopherol were also tested for antioxidant activity using the same liposome system. Lipid oxidation was initiated by iron redox cycling by adding 0.1 mL of 50 mM $FeCl_3$ and 0.1 mL of 10 mM ascorbate into the liposome/protein solution (6 mL). Samples were incubated in a 37 °C water bath for 1 h, and lipid oxidation was immediately determined as described below.

Peroxide Value (PV). The PV was measured according to the AOCS standard procedure (*26*). Specifically, 1 mL of liposome oxidation sample was mixed with 10 mL of acetic acid/chloroform solution (3:2). The slurry was gently swirled, and 0.3 mL of saturated potassium iodide solution was then added. After 1 min of reaction with occasional shaking, 30 mL of distilled H_2O and 0.3 mL of 0.1% starch solution were added. The mixed solution was titrated with 2 mM sodium thiosulfate ($Na_2S_2O_3$) until the intense blue color disappeared. A control blank (without liposome) was also assayed. The PV was calculated as

$$\mathrm{PV\ (mequiv/L)} = [(S - B) \times C]/V_{\mathrm{s}}$$

where S and B are the volumes (mL) of sodium thiosulfate solution consumed by the sample and by the blank, respectively, C is the concentration (mM) of sodium thiosulfate solution, and V_{s} is the sample amount (1 mL).

Thiobarbituric Acid-Reactive Substances (TBARS). The concentration of TBARS (secondary products from lipid oxidation) was determined according to the method of Sinnhuber and Yu (*27*) with slight modifications as described by Wang and Xiong (*28*). After reaction with TBA, the sample solution was mixed with chloroform (1:1 ratio, v/v), vortexed, and subsequently centrifuged at 1800*g* for 10 min. The TBARS value, expressed as milligrams of malonaldehyde per liter of liposome oxidation sample solution, was calculated using the equation

$$\mathrm{TBARS\ (mg/L)} = (A_{532}/V_{\mathrm{s}}) \times 9.48$$

where A_{532} is the absorbance (532 nm) of the assay solution, V_{s} is the volume of liposome oxidation sample solution (1 mL), and 9.48 is a constant derived from the dilution factor and the molar extinction coefficient (152 000 M^{-1} cm^{-1}) of the red, TBA reaction product.

Reducing Power. The reducing ability of hydrolyzed zein was measured using the ferric reducing/antioxidant power (FRAP) assay (*29*). In the assay, 3.0 mL of freshly prepared FRAP reagent (mixture of 2.5 mL of 10 mM TPTZ, 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$, and 25 mL of 30 mM acetate, pH 3.6) was warmed to 37 °C, and 0.1 mL of hydrolyzed zein, along with 0.3 mL of H_2O, was then added. For comparison, 0.1 mL of 0.001% BHA or 0.01% ascorbate was used. Absorbance (593 nm) of samples as well as blank solutions was taken

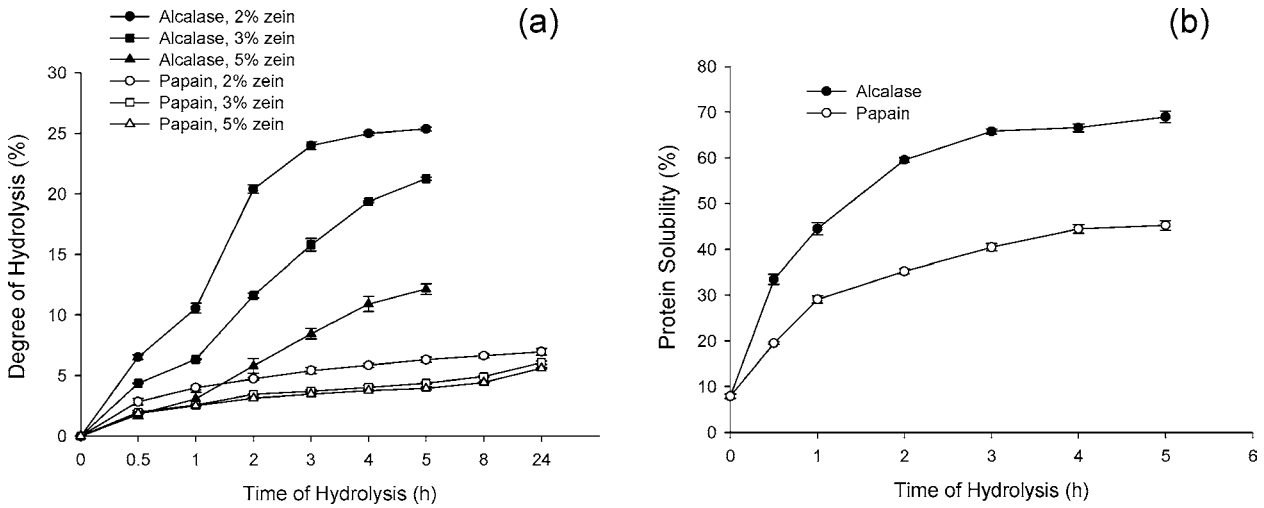

Figure 1. Degree of hydrolysis (**a**) and solubility (**b**) of alcalase- and papain-hydrolyzed zein. Protein solubility was measured with a 3% zein solution.

at 30 s intervals for up to 8 min. Sample FRAP values were calculated on the basis of an $FeSO_4$ standard curve (prepared with 100−1000 μM $FeSO_4 \cdot 7H_2O$) and were expressed as $FeSO_4$ equivalent (μM).

Radical-Scavenging Activity (RSA). The analysis was performed using an ABTS decolorization assay (*30*). RSA of protein samples was measured by mixing 10 μL of the samples with 990 μL of diluted $ABTS^{\bullet+}$ that was generated by reacting ABTS stock solution (7 mM) with 2.45 mM (final concentration) potassium persulfate. For comparison, 10 μL of 0.001% BHA or 0.01% ascorbate was used. The absorbance reading (734 nm) was taken at 1 min intervals for a total of 8 min. A standard RSA curve was prepared by reacting 10 μL of a series of concentrations of Trolox (0.05, 0.10, 0.25, 0.50, 1.0, 1.5, and 2.0 mM) with 990 μL of diluted $ABTS^{\bullet+}$ solution for 1 min. The degree of RSA of the protein samples was calculated on the basis of the Trolox standard curve and was expressed as Trolox equivalent antioxidant capacity (TEAC, mM).

Metal-Chelating Activity. The procedure described previously was followed to determine the Cu^{2+} and Fe^{2+} chelation activities of zein hydrolysates (*28*). In the Cu^{2+} chelation assay, 1 mL of 2 mM $CuSO_4$ was mixed with 1 mL of 10% pyridine and 20 μL of 0.1% pyrocatechol violet. After the addition of 1 mL of zein hydrolysates, the disappearance of the blue color, due to dissociation of Cu^{2+}, was monitored by determining the absorbance at 632 nm. For comparison, 1 mL of 0.001% BHA or 0.01% ascrobate was used. In the Fe^{2+} chelation assay, 1 mL of 20 μM $FeCl_2$ was mixed with 1 mL of 0.5 mM ferrozine, which produces a chromaphore that absorbs strongly at 562 nm. After the addition of 0.5 mL of zein hydrolysates, the absorbance change, due to dissociation of Fe^{2+}, was measured at 562 nm. The metal ion-chelating activity by zein hydrolysates was calculated as follows:

$$[1 - (\text{sample solution absorbance}/\text{blank solution absorbance})] \times 100$$

Statistical Analysis. Three independent trials were conducted to test the antioxidant activities of zein hydrolysates, with each separate trial using a new batch of zein hydrolysates. All of the specific antioxidant assays were carried out in triplicate. Data were analyzed using the General Linear Models procedure of the Statistix 7.0 software package (Analytical Software, St. Paul, MN) for microcomputers. Analysis of variance (AOV) was done to determine the significance of the main effects. Significant differences ($P < 0.05$) between means were identified using least significant difference (LSD) procedures.

RESULTS

DH and Protein Solubility. The DH of zein ranged from 1.8 to 25.4%, depending on the enzymes used, the time of hydrolysis, and substrate concentration (**Figure 1a**). As expected, the DH increased with the incubation time. The 5-h DH values of 2, 3, and 5% substrate concentrations were, respectively, 25.4, 21.3, and 12.1% for alcalase and 6.3, 4.4, and 3.9% for papain. The inverse relationship between DH and the substrate concentration probably reflected a reduced substrate and enzyme diffusivity as the solid content increased. The DH of alcalase-hydrolyzed zein was higher ($P < 0.05$) than that of papain-hydrolyzed zein for comparable hydrolysis times. Even when the time of hydrolysis was extended to 24 h, the DH of papain-hydrolyzed zein reached only 5.6−7.0%. Although the 2% substrate concentration had the highest DH, it would still be less efficient than the 3% substrate concentration (substrate conversion per unit of enzyme). Hence, all of the subsequent experiments were conducted with a 3% zein concentration.

Solubility of zein in the aqueous solution increased with DH, which also rose with hydrolysis time (**Figure 1b**). Nonhydrolyzed zein was barely soluble in water, but its solubility increased rapidly following hydrolysis, reaching 69.0 and 45.2% after 5 h of hydrolysis by alcalase and papain, respectively. The higher ($P < 0.05$) level of DH and protein solubility by alcalase treatment suggested that alacalse has a higher affinity and, therefore, is a more efficient enzyme choice than papain for preparing zein hydrolysates. As more charged groups (i.e., NH_3^+ and COO^-) became available from peptide cleavage, there was an enhanced protein−water interaction and a stronger electrostatic repulsion between peptides and, thus, an improved protein solubility (*22*, *28*).

Degradation of Zein Subunits. The electrophoretic patterns of the alcalase-hydrolyzed zein are shown in **Figure 2**. Nonhydrolyzed zein was composed largely of three polypeptides (bands II, III, and IV). These zein components matched the MW of those reported in the literature (*31*), that is, γ-zein (27 kDa) for band II and α-zein (21−25 kDa) for bands III and IV. All three major zein proteins were susceptible to alcalase, showing a steady degradation over time (**Figure 2a**). Within 0.5 h, the band intensity (peak area) had decreased ($P < 0.05$) from 0.78 to 0.47 for band I, from 0.74 to 0.62 for band II, and from 1.58 to 1.01 for band III (**Table 1**). After 5 h, only trace amounts of the proteins were left. Although these three major protein subunits were readily hydrolyzed, the band IV polypeptide was degraded most rapidly and essentially vanished by 3 h of hydrolysis.

However, degradation of the major zein proteins as well as the minor ones in samples hydrolyzed for 30 min or longer did not result in any short peptides that could be observed in the polyacrylamide gel. Therefore, hydrolysates were subsequently sampled at shorter time intervals (every 5 min) and subjected

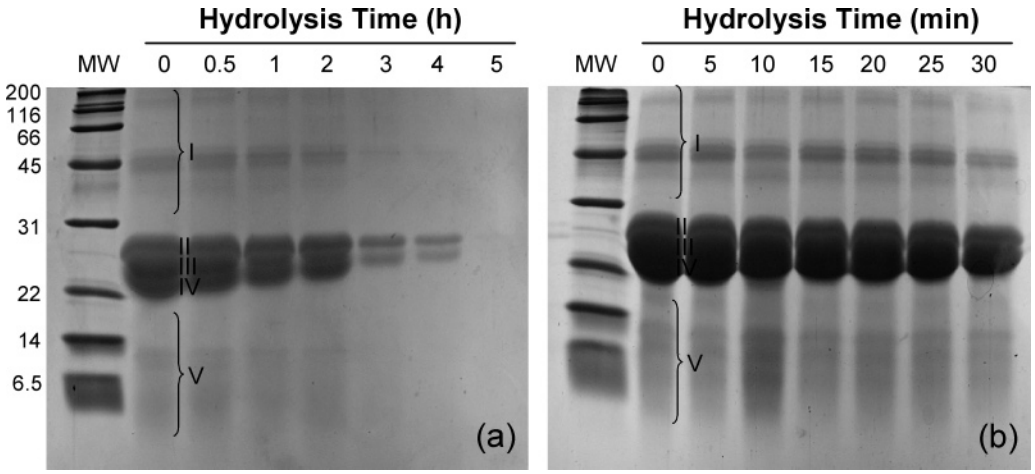

Figure 2. SDS-PAGE of alcalase-hydrolyzed zein (3%): (**a**) 0–5 h of hydrolysis; (**b**) 0–30 min of hydrolysis. II, γ-zein (27 kDa); III, α-zein subunit 1 (25 kDa); IV, α-zein subunit 2 (21 kDa). Bands I and V are groups of unidentified proteins. Numbers by the molecular weight (MW) lane are in kDa. To each lane was loaded a 20-μL sample (4.6 μg/μL protein for gel **a** and 7 μg/μL protein for gel **b**).

Table 1. Protein Band Intensity in Zein during Alcalase Hydrolysis up to 5 h[a]

	hydrolysis time						
band	0 h	0.5 h	1 h	2 h	3 h	4 h	5 h
I	0.78a	0.47ab	0.38a,b	0.30a,b	0.20b	0.18b	0.14b
I	0.74a	0.62ab	0.61ab	0.53b	0.39b,c	0.28c	0.07d
III	1.58a	1.01b	0.58c	0.50c	0.28d	0.19d,e	0.06e
IV	1.49a	1.20b	0.89c	0.71c	0.09d	0.03d	0.02d
V	0.80a	0.67a,b	0.44a,b	0.54a,b	0.27a,b	0.25b	0.21b
total	5.72a	3.79b	2.90bc	2.58c	1.23d	0.87d	0.50d

[a] Protein band intensity was measured by scanning the SDS-PAGE gel and was expressed as the height of peak × width of the peak at half-height. Means in the same row with different letters differ significantly ($P < 0.05$).

to SDS-PAGE for the detection of possible transit peptides. However, the result also showed no distinct short peptides in the 6−30 kDa MW region other than some faint smearing that appeared (**Figure 2b)**. Alcalase is an endoproteinase that severs proteins by cleaving the internal peptide bonds. Therefore, it appeared that the intermediate fragments derived from γ-zein and α-zein were highly susceptible to alcalase and were extremely short-lived. Pena-Ramos and Xiong (*11*) studied alcalase hydrolysis of soy proteins and showed a major degradation product (≈45 kDa) produced from β-conglycinin, but essentially not any other shorter peptides. The protein hydrolysates were likely a mixture of extremely small peptides. Chromatographic determination of alcalase-hydrolyzed whey protein revealed numerous oligopeptides with MW estimated to be <1 kDa and between 1 and 2.5 kDa (*8*). Doucet et al. (*32*) noted that of the 130 peptides identified in alcalase-hydrolyzed whey proteins, >80% were small peptides with MW of <2 kDa.

Amino Acid Composition. Nonhydrolyzed zein had a relatively high content of glutamine/glutamic acid and nonpolar amino acids such as leucine, alanine, and proline (**Table 3**). The overall amino acid composition was similar to that reported in the literature (*33*, *34*). Hydrolysis with alcalase did not appreciably change the percentage of most amino acids in the soluble fractions of the hydrolysates. However, both sulfur amino acids (Cys and Met) had 2−3-fold increases in hydrolyzed samples. A slight increase in lysine and valine was also noted in hydrolyzed zein.

Inhibition of Lipid Oxidation in a Liposome System. The zein hydrolysates prepared by both alcalase and papain exhibited significant inhibition of lipid peroxide formation, and the inhibition was more pronounced as the hydrolysis time was prolonged until ≈4 h (**Figure 3**). However, alcalase-hydrolyzed zein exhibited stronger ($P < 0.05$) antioxidant activity than papain-hydrolyzed zein at any of the hydrolysis times. The PV of the zein-free blank control (PV = 3.73 mequiv/L) was lowered by 21.4, 32.2, 33.8, 43.7, 57.1, and 46.4% by zein following 0.5, 1, 2, 3, 4, and 5 h of hydrolysis with alcalase, compared to 12.0, 19.1, 21.8, 24.5, 31.1, and 31.9% by papain-hydrolyzed zein prepared with the same hydrolysis times. Nonhydrolyzed zein, which was predissolved in 65% ethanol, also showed a slight inhibitory effect on PV formation in the liposome system.

Because the enzyme treatments markedly improved zein solubility, the observed antioxidant activity enhancement could

Table 2. Comparison of Antioxidative Activities between Alcalase-Hydrolyzed Zein and Several Common Antioxidants[a]

	BHA[b] (0.01%/0.001%)	α-tocopherol (0.01%)	ascorbate (0.01%)	nonhydrolyzed zein[c] (0.5%/3%)	hydrolyzed zein[c] (0.5%/3%)
PV (mequiv/L)	1.13c	1.53b		3.23a	1.60b
TBARS (mg/L)	0.21c	0.32b		0.78a	0.23c
FRAP (μmoL/L, $FeSO_4$)	3965a		1010b	188d	874c
TEAC (mmol/L, Trolox)	2.90a		0.62c	0.49c	2.44b
Cu^{2+}-chelating activity (%)	0.00c		7.26b	4.08b	59.26a

[a] Means in the same row with different letters differ significantly ($P < 0.05$). [b] The concentrations of BHA (butylated hydroxyanisole) were 0.01% for TBARS, PV, and chelating activity tests and 0.001% for FRAP and TEAC tests. [c] The concentrations of nonhydrolyzed and hydrolyzed zein (4 h) were 0.5% for TBARS and PV tests and 3% for FRAP, TEAC, and Cu^{2+}-chelating activity tests.

Table 3. Amino Acid Composition (Percent) of Zein and Zein Hydrolysates[a]

amino acid[b]	0 h (intact)	0.5 h	1 h	2 h	4 h
cysteine	0.11	0.30	0.32	0.31	0.22
isoleucine	2.9	3.26	3.19	2.73	3.11
aspartic acid	4.8	4.75	4.70	4.78	4.76
leucine	18.8	17.89	17.61	17.84	18.25
threonine	2.44	2.44	2.46	2.38	2.48
tyrosine	5.05	4.88	4.86	5.03	5.04
serine	5.10	4.79	4.83	5.03	4.92
phenylalanine	7.01	6.70	6.66	6.69	6.83
glutamic acid	22.71	22.46	22.22	22.68	23.29
lysine	0.12	0.17	0.16	0.14	0.14
glycine	1.03	1.22	1.29	1.21	1.04
alanine	9.03	8.75	9.12	9.29	8.80
histidine	1.12	1.09	1.11	1.01	1.11
valine	2.87	3.31	3.57	3.24	3.18
arginine	1.23	1.26	1.23	1.19	1.26
methionine	0.83	2.22	2.18	2.09	1.77
proline	13.13	12.93	12.94	12.84	12.72
total	100.00	100.00	100.00	100.00	100.00

[a] Zein hydrolysate samples are the supernatants (soluble) of the hydrolysates obtained from 0.5, 1, 2, and 4 h of alcalase hydrolysis. [b] The aspartic acid and glutamic acid contents include, respectively, asparagine and glutamine.

have resulted from the increased concentration of soluble antioxidative peptides. To determine whether the concentration of soluble protein was a sole determinant factor, the PV reduction per unit weight of both total protein and the soluble fractions (mequiv/L per g of protein) was calculated. As displayed in **Figure 3b**, the "total protein" and "soluble protein" lines ran almost parallel over the entire range of hydrolysis time for both enzyme treatments, suggesting that the PV reduction by mixed zein hydrolysates was indeed attributed to soluble proteins (peptides). However, even at the same soluble protein concentration levels, papain-hydrolyzed zein still tended to have a lesser effect than alcalase-hydrolyzed zein. Therefore, it can be postulated that the specific short peptides or the peptide/amino acid composition in the latter hydrolysates predisposed the liposome lipids to a reduced vulnerability to oxidative attack. Furthermore, the somewhat zigzag-shaped PV reduction line, exhibited by the alcalase-produced soluble zein protein, could be viewed as a peptide composition effect, that is, the specific peptide composition produced at a particular hydrolysis time may be more antioxidative, independent of the protein concentration. For alcalase treatment, the antioxidant activity of zein appeared to reach a maximum level following 4 h of hydrolysis.

The effect of enzyme hydrolysis on the ability of zein to inhibit TBARS formation in the liposome system was strikingly similar to that from the PV analysis (**Figure 4**), suggesting that lipid peroxides generated in the oxidizing liposome system were readily decomposed into malondialdehyde or other secondary, TBA-reactive compounds. For alcalase-hydrolyzed zein, TBARS were lowered by 38.3, 45.0, 50.9, 58.3, 74.7, and 74.4% at 0.5, 1, 2, 3, 4, and 5 h of hydrolysis, when compared to the zein-free blank. For papain-hydrolyzed zein, 14.7, 23.0, 34.1, 45.8, 52.7, and 56.9% less TBARS were produced for the same hydrolysis periods. Even with 24 h of hydrolysis, the TBARS level of papain-hydrolyzed zein was still higher ($P < 0.05$) than that of 5-h alcalase-hydrolyzed zein. Furthermore, whereas the TBARS reduction per gram of soluble protein increased continuously with hydrolysis time in the papain system, that of the alcalase-hydrolyzed samples exhibited a nonlinear pattern, with a maximum value being observed in the 4-h hydrolysate.

The total protein concentration effect was further assessed by using the 4-h hydrolysates produced with alcalase. As expected, the degree of inhibition of both PV and TBARS increased with the application level of the zein hydrolysates (**Figure 5**). Because of its relatively high antioxidant efficacy, alcalase-hydrolyzed zein was further evaluated for possible antioxidant mechanisms. A 3% protein concentration (unless specified otherwise) was used in the subsequent analyses, although the cost-effectiveness of this application level must still be evaluated.

Reducing Power. The FRAP value of alcalase-hydrolyzed zein increased with the hydrolysis time ($P < 0.05$) (**Figure 6a**). The 4-min FRAP value of nonhydrolyzed zein was 188 μM, and those of the hydrolysates were 2−5-fold greater ($P < 0.05$). The 3- and 4-h hydrolysates were further analyzed at different protein concentrations. The FRAP value increased almost linearly with the protein concentration ($P < 0.05$), although the rate of increase was slower at higher protein concentrations (**Figure 6b**).

RSA. The RSA of zein, expressed as TEAC, increased drastically after hydrolysis for 0.5 h (**Figure 7a**). However, there were only small changes in RSA upon further hydrolysis. Reaction of peptides with the $ABTS^{\bullet+}$ radical was time de-

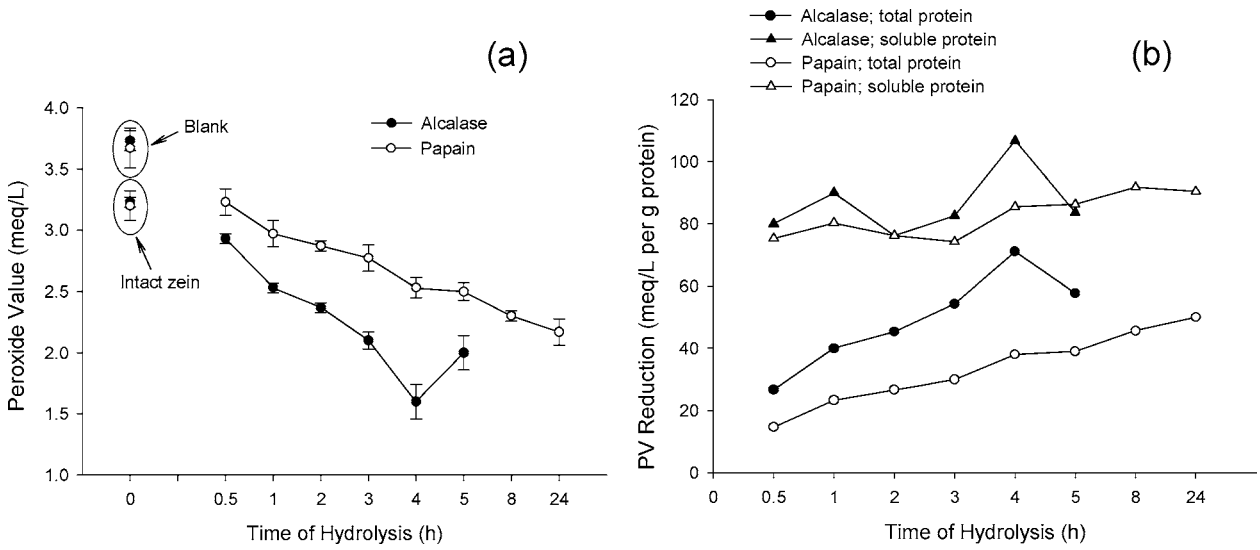

Figure 3. Inhibition of peroxide formation by hydrolyzed zein (3%) in a liposome-oxidizing system: (**a**) inhibition by the whole zein hydrolysates produced at different hydrolysis times; (**b**) calculated PV reduction per gram of total protein or gram of soluble protein corresponding to different hydrolysis times.

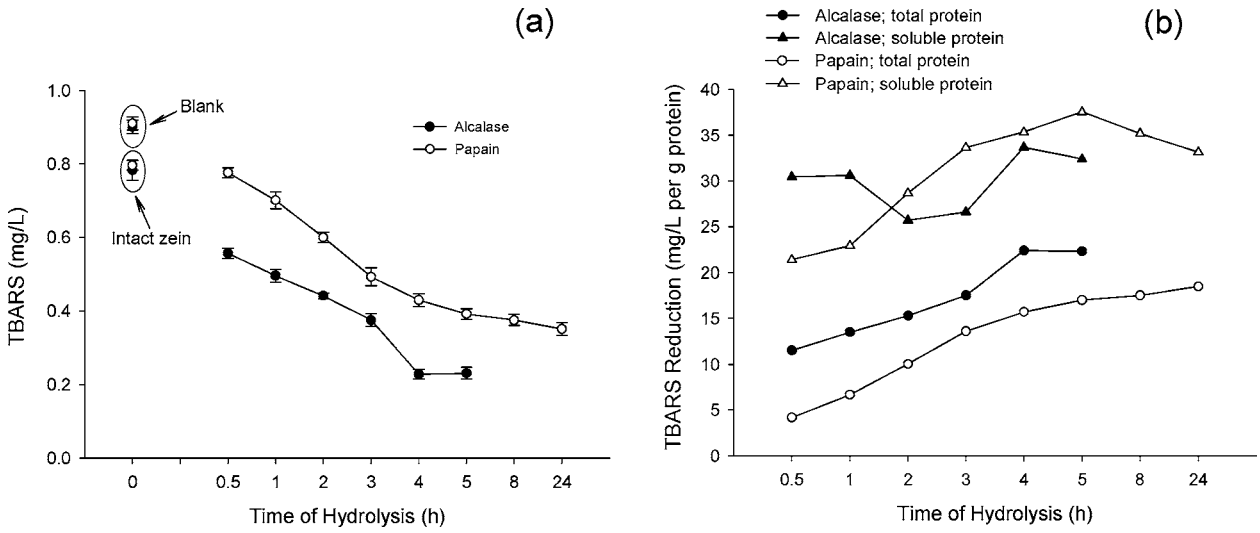

Figure 4. Inhibition of TBARS formation by hydrolyzed zein (3%) in a liposome-oxidizing system: (**a**) inhibition by the whole zein hydrolysates produced at different hydrolysis times; (**b**) calculated TBARS reduction per gram of total protein or gram of soluble protein corresponding to different hydrolysis times.

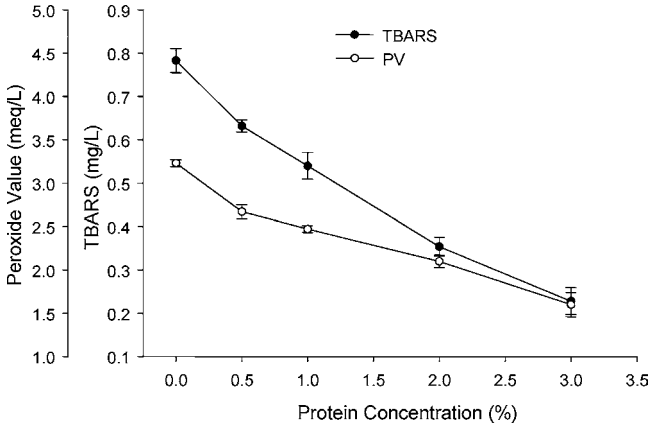

Figure 5. Inhibition of PV and TBARS production in a liposome-oxidizing system by alcalase-hydrolyzed zein (4 h of hydrolysis) at various total protein concentrations.

pendent, and a minimum of 4 min was required before a plateau was established. Samples of nonhydrolyzed zein, which was predissolved in 65% ethanol, also had some radical-scavenging ability, but its activity was far less than that of its hydrolyzed products. The 3- and 4-h hydrolysates were further assayed to determine the protein concentration effect. There were negligible differences between 1, 2, and 3% protein concentrations (**Figure 7b**). The result suggested that hydrolyzed zein was effective and efficient in quenching radicals, and, under the RSA assay conditions used, zein hydrolysate at a concentration of 1% (or possibly even lower) was adequate to stabilize oxidizing radicals.

Metal Ion Chelation. Nonhydrolyzed zein was virtually incapable of sequestering either copper (Cu^{2+}) or ferrous (Fe^{2+}) ions. However, the Cu^{2+} chelation activity of zein was markedly increased following hydrolysis, for example, from negligible (4%) (control) to 68% after 5 h of hydrolysis (**Figure 8a**). In contrast, the Fe^{2+} chelation activity of the protein sample, which was almost nonexistent (2%), increased only to 7% after 5 h of hydrolysis. The protein concentration effect test showed a marked enhancement of the Cu^{2+} chelation activity of the zein hydrolysates; however, the same protein concentration increase did not bring about significant improvement in the hydrolysates' Fe^{2+}-binding ability (**Figure 8b**).

Comparison with Common Antioxidants. The antioxidant activities of intact and alcalase-hydrolyzed zein were compared with those of several benchmark antioxidants (BHA, α-tocopherol, and ascorbate) in the liposome-oxidizing system. As summarized in **Table 2**, a 0.5% hydrolyzed zein sample had a PV inhibitory effect similar to that of 0.01% α-tocopherol and a TBARS inhibitory effect similar to that of 0.01% BHA ($P > 0.05$). The 0.001% BHA and the 0.01% ascorbate solutions had significantly higher ($P < 0.05$) FRAP values than 3% hydrolyzed zein, indicating stronger hydrogen donation capability by these commercial antioxidants. The 3% zein hydrolysate showed a slightly lower radical-scavenging activity than 0.001% BHA, but it was much more effective ($P < 0.05$) than 0.01% ascorbate. Furthermore, hydrolyzed zein at a 3% concentration level was far superior to ascorbate or BHA in sequestering Cu^{2+}, suggesting its potential as an effective antioxidant in foods that are rich in this prooxidative metal ion.

DISCUSSION

The literature reports on zein as an antioxidant substance are largely related to the physical barrier established by zein proteins. By forming an air-resistant coating, zein-treated foods are separated from molecular oxygen. Furthermore, zein can interact with nonpolar groups of fatty acids and lipids to inhibit oxidation (*35*). Dried zein (powder) was found to inhibit methyl linoleate ester oxidation, but the effect was largely attributed to the traces of unextracted phenolics and, to a lesser extent, to the physical shield that zein formed around lipids (*36*).

The ability of zein hydrolysates to chemically inhibit lipid oxidation as demonstrated in the present study was influenced by the DH. Because of its compact structure, intact maize zein had a minimal antioxidant activity. Disruption of the native zein structure by enzyme hydrolysis resulted in the opening and exposure of active amino acid residues and patches capable of reacting with oxidants. The increase in the soluble antioxidative protein fragments (peptides) appeared to be a main cause for the antioxidant activity improvement. However, the lack of a direct relationship between antioxidant activity and protein solubility (or DH) suggested that the specific composition (e.g., type of peptides, ratio of different freed amino acids) was an important factor as well. Alcalase is a serine endopeptidase with

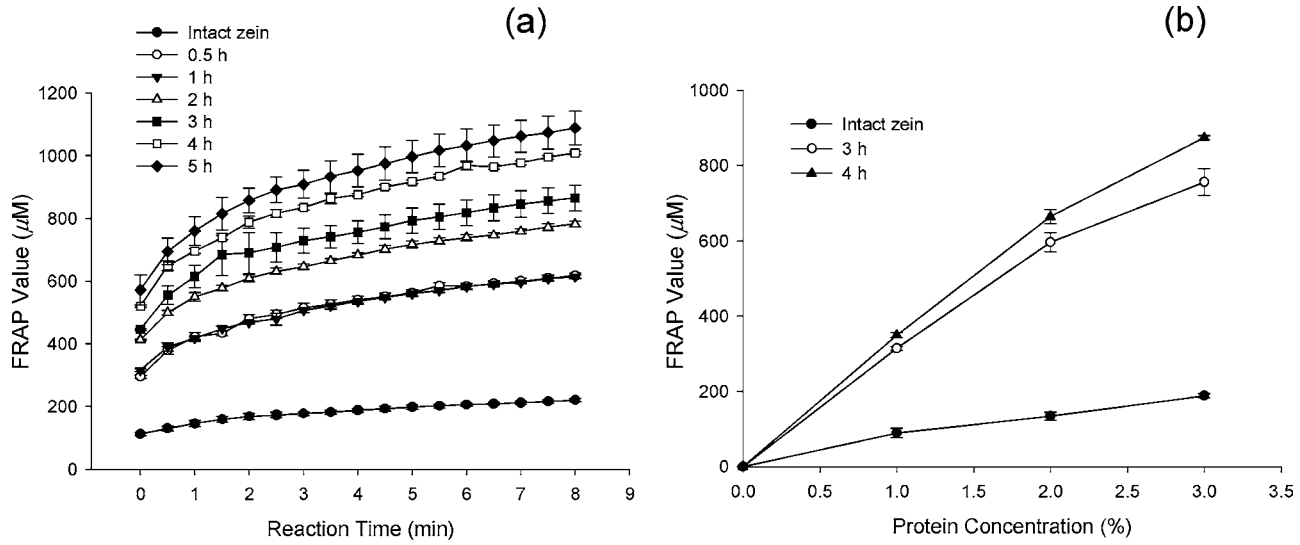

Figure 6. Effect of hydrolysis time (**a**) and protein concentration (**b**) on the FRAP value of alcalase-hydrolyzed zein. The FRAP assay in (**a**) was based on a 3% zein solution, and the FRAP value in (**b**) represented the 4-min assay values.

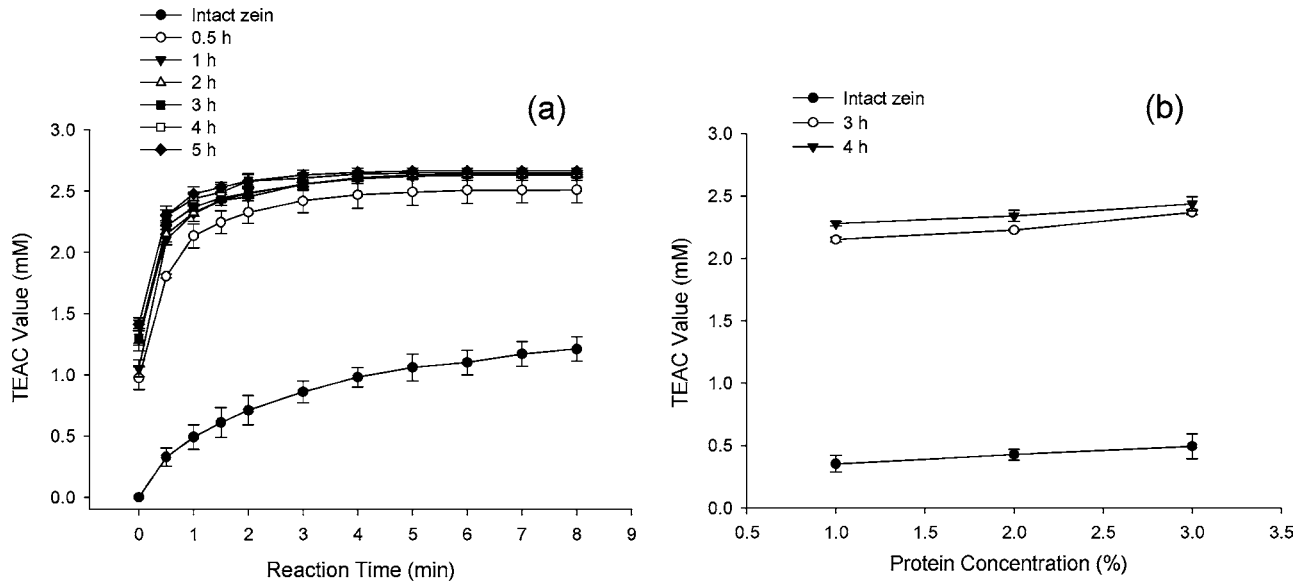

Figure 7. Effect of hydrolysis time (**a**) and protein concentration (**b**) on the radical scavenging activity of alcalase-hydrolyzed zein. The activity was expressed as TEAC. The TEAC assay in (**a**) was based on a 3% zein solution, and the TEAC in (**b**) represented the 4-min assay values.

a broad specificity but a preference for peptide bonds next to large, uncharged amino acid side-chain groups (*32*). Cleavages of peptides by alcalase ostensibly increased cysteine-, methionine-, lysine-, and valine-containing zein fragments or oligopetides that were sufficiently hydrophilic to be soluble in the aqueous solution. These particular amino acid residues have previously been shown to exert antioxidant activity (*37*, *38*).

Chen et al. (*39*) reported that the antioxidant activity of soy protein hydrolysates was dependent upon the characteristic amino acid sequences of the peptides derived, which was dictated by the protease specificity. Hirose and Miyashita (*40*) postulated that beside acting as a free radical scavenger, a protein hydrolysate could serve as a protecting membrane surrounding lipid droplets against oxidation initiators. Presumably, by converting to more amphoteric and structurally flexible short peptides, zein fragments could readily diffuse to the water−oil interface, where they would adsorb or loosely bind to the phospholipids membrane in the liposome where oxidation occurred. Conversely, if the hydrolysis became too extensive, for example, >4 h with alcalase as shown in this study, it could reduce the peptide's ability to act as a physical barrier to prevent oxidants from reaching the lipid fraction in the liposome. Wu et al. (*13*) found that a peptide from fish muscle hydrolysates with a 1.4 kDa MW possessed stronger antioxidant activity than a 0.9 or 0.2 kDa peptide.

The enhanced antioxidant activity of the soluble fraction of zein hydrolysates with hydrolysis time on the same weight basis (PV and TBARS reductions per gram of soluble protein) was strong evidence that small MW fragments and amino acids derived from zein were probably preferred targets of peroxide radicals in the oxidizing liposome system. As shown in this study, as well as from previous analysis (*33*), zein is made up of a large amount of hydrophobic amino acid residues, including leucine, proline, alanine, and phenylalanine in addition to glutamine. The presence of these nonpolar amino acids, particularly proline and leucine, has been correlated to the antioxidant activity of hydrolyzed proteins in other model systems (*8*, *39*). The results from the present study also demonstrated that hydrolysis with alcalase increased the relative content of other antioxidative amino acids, such as cysteine,

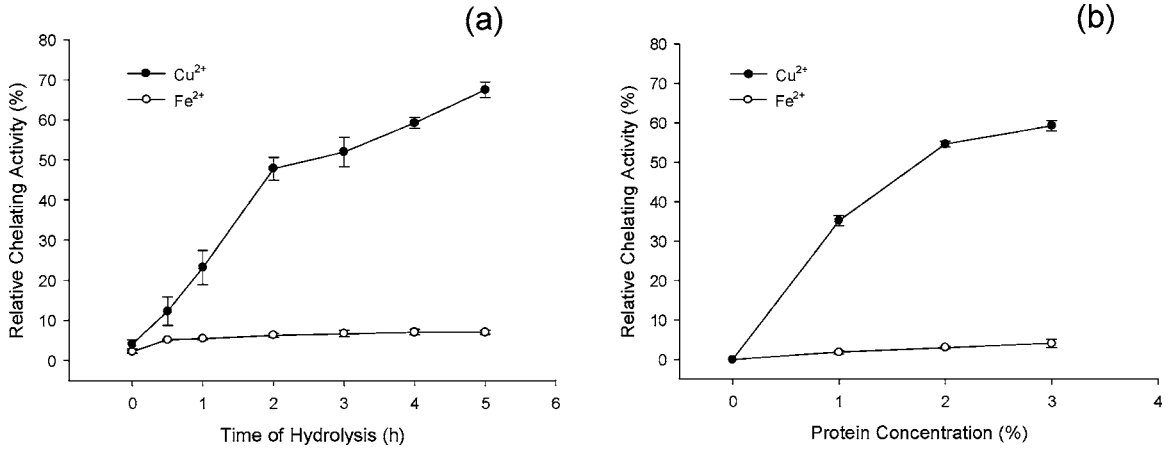

Figure 8. Effect of hydrolysis time (**a**) and protein concentration (**b**) on copper (Cu^{2+})- and iron (Fe^{2+})-chelating activity of alcalase-hydrolyzed zein. The assay in (**a**) was based on a 3% protein concentration, and the assay in (**b**) was based on the 3-h zein hydrolysate.

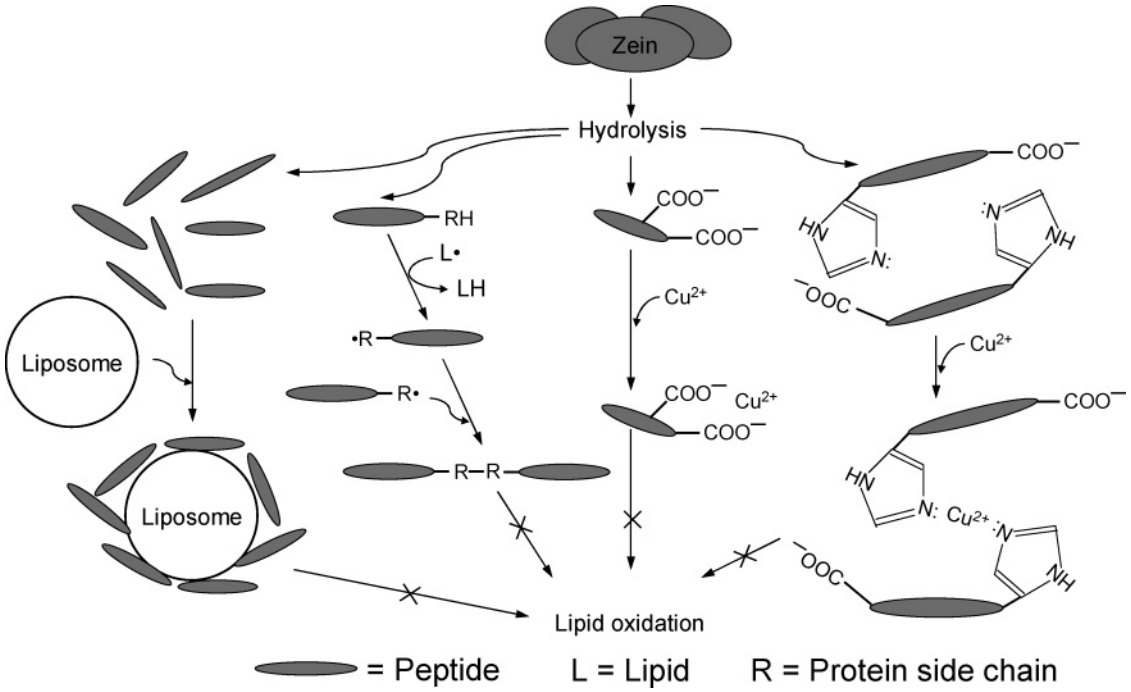

Figure 9. Plausible modes of inhibition of lipid oxidation by hydrolyzed zein.

methionine, and valine, which may help to explain the inhibition of oxidation in liposome systems by the added zein hydrolysates.

The antioxidant activity demonstrated by hydrolyzed zein appeared to involve complex modes of actions. In addition to the plausible physical effect (shielding), results from the FRAP, TEAC, and ferrozine tests indicated the involvements of chemical mechanisms that are more commonly observed, that is, electron/hydrogen donation, radical quenching, and metal ion chelation. Inhibition of lipid oxidation by some di- or tripeptides in oil or metal-catalyzed liposome suspensions has been attributed to chelation of prooxidative metal ions and termination of free radical chain reactions (*41*, *42*). The inhibition was accomplished either through the specific amino acid residue side-chain groups or through the specific peptide structure. The strong reducing power of hydrolyzed zein may be attributed to the increased availability of hydrogen ions (protons and electrons) due to peptide cleavages. It was shown that blocking the N-terminal and C-terminal of branched-chain amino acid residues greatly diminished the antioxidant activity of peptides (*43*), suggesting that both terminals play a role in inhibiting lipid oxidation (oxygen uptake). Likewise, the increased radical-scavenging ability of hydrolyzed zein likely resulted from structural changes leading to a greater radical quenching capability. Saito et al. (*42*) reported that available tyrosine at the C terminus is critical to radical scavenging by some peptides. Compared to lipid radicals, protein radicals tend to be long-lived and more stable (*44*).

Transitional metal ions, such as Fe^{2+} and Cu^{2+}, can catalyze the generation of reactive oxygen species, for example, hydroxyl radical ($^{\bullet}OH$) and superoxide radical ($O_2^{\bullet-}$), which, in turn, oxidize unsaturated lipids (*45*, *46*). Metal prooxidant sequestering has been observed in many short peptides, such as carnosine (*47*), fragments of myofibrillar proteins (*18*), and mixed potato peptides (*28*). Presumably, peptide cleavages led to an enhanced Cu^{2+} binding due to an increased concentration of carboxylic groups (COO^-), thus removing the prooxdative free metal ion from the liposome system. The direct relationship between soluble protein/peptide concentration and the increase in the chelation capability supported this premise. Chelation of Cu^{2+} in hydrolyzed zein could also result due to increased exposures of certain amino acids, such as histidine, which is a well-known metal-binding amino acid, or a protein residue (*48*−*50*). The

cause for the poor Fe^{2+} binding by hydrolyzed zein was not clear, but it may be related to the greater number of coordination sites present in it (thus, more chelators are required) when compared with Cu^{2+}. Haga et al. (*51*) also noted a far weaker Fe^{2+} binding than Cu^{2+} binding (a 875-fold difference) by dehydrin, a protein found in higher plants and yeast.

Despite the strong antioxidative activity demonstrated by the zein hydrolysates prepared under different conditions, they were generally not as effective as the phenolic antioxidant BHA (except for the Cu^{2+}-binding activity). This may be explained because zein hydrolysates could contain both antioxidative and prooxidative components (peptides, amino acids), rendering them less efficient against lipid oxidation under certain particular conditions (*7*−*10*).

In conclusion, controlled hydrolysis by alcalase and papain enhanced the antioxidant activity of maize zein in a liposome model system. The activity was attributed to the increased protein solubility and the composition of specific peptides/amino acids present in the zein hydrolysates. Further analyses suggested the possible involvement of multiple reactions and processes leading to the inhibition of lipid oxidation, that is, the ability to donate hydrogen, to stabilize or terminate radicals, to sequester prooxidative metal ions, and probably also to form a physical barrier around the fat droplets (**Figure 9**). Thus, enzyme-hydrolyzed zeins, particularly those with alcalase, may serve as useful natural antioxidants to preserve the quality and extend the shelf life of foods. Further studies are needed to identify the specific peptides in zein hydrolysates that are responsible for the overall antioxidative capability.

ABBREVIATIONS USED

DH, degree of hydrolysis; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PV, peroxide value; TBARS, thiobarbituric acid-reactive substances; FRAP, ferric reducing/antioxidant power; RSA, radical-scavenging activity; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; TEAC, Trolox equivalent antioxidant capacity; BHA, butylated hydroxyanisole.

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